STUDIES ON THE EFFECTS OF IRRADIATION OF CELLULAR PARTICULATES.[1] III. THE EFFECT OF COMBINED RADIATION TREATMENTS ON PHOSPHORYLATION

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Previous work has established that particulate-linked, oxidative phosphorylation is very sensitive to ionizing radiation. This is true whether the radiation is delivered to the whole organism (van Bekkum and Vos, 1955) or to the isolated particulates (Yost and Robson, 1957). These findings suggest that one of the principal modes of action of radiation is the destruction of the cells' ability to carry on their "energy metabolism." Interference with oxidative phosphorylation would be expected to have profound effects upon all reactions of the cell which are dependent upon a supply of ATP (the synthesis of proteins and nucleic acids being the most obvious). This suggests that some of the observed damage to the chromosomes resulting from radiation might be the result of extra-nuclear damage to the phosphorylating mechanism, which suggestion is supported by the observation of many investigators (see Wolff and Luippold, 1955) that the number of chromosomal aberrations recovered after radiation treatment is altered by known inhibitors of oxidative phosphorylation.

The radiation literature contains many references to the effects of combined radiations in producing alterations in chromosomal aberration (Giles, 1954). It has been shown that pretreatment with ultraviolet radiation will cause a *decrease* in the number of translocations recovered after exposure to ionizing radiations (Swanson, 1943). On the other hand, pretreatment with near infrared radiation causes an increase in the recovered aberrations resulting from exposure to ionizing radiations (Swanson, 1949). To date, no completely satisfactory explanation has been advanced for these results. Explanations of complimentary or antagonistic action by radiations used in combination are complicated by the duration of these effects. In the case of infrared treatment, there may be a time lapse of at least 96 hours between the application of the infrared and the x-rays with no decrease in effectiveness. Thus, the condition induced is stable, although it may be altered by drastic environmental changes (Swanson and Yost, 1951). However, when the data from combined radiation studies are taken together with the data on the effects of combined radiation and chemical treatments (Kihlman, Merz and Swanson, 1957), an hypothesis may be formulated. It is possible that the various combined treatment effects are the result of activation or inactivation (depending upon the radiations or chemicals used) on oxidative phosphorylation.

The data presented in this paper are the result of studies designed to test the hypothesis that the observed effects of combined radiation treatments are the result

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of interference with the normal phosphorylation pathways. The effect of ultraviolet radiation alone and in combination with gamma radiation, and the effect of infrared radiation alone and in combination with gamma and ultraviolet radiations was studied. Isolated particulates from rat liver were chosen since the effects of gamma radiation and some ultraviolet effects were already known (Yost and Robson, 1957; Yost, Robson and Spiegelman, 1956).

Materials and Methods

White laboratory rats were starved overnight and sacrificed by a blow on the head. The liver was removed and placed in cold 0.85% KCl, where much of the blood is washed free. The liver was weighed and pressed through a bronze screen to remove connective tissue. The resulting mash was then suspended in 50 ml. of cold 8.5% sucrose containing 0.005 *M* disodium versenate and homogenized in a glass homogenizer. The mitochondria were then separated from the rest of the homogenate by differential centrifugation (Schneider, 1948). The mitochondrial fraction alone was kept. The final centrifugate was re-suspended in 1 ml. 8.5% sucrose per gram of original liver. All dilutions were made from this stock solution.

For treatment with gamma radiation the preparation was diluted 1 in 20 with cold distilled water. This diluted suspension was radiated in a 25-ml. "Lusteroid" centrifuge tube in the beam of a 440 curie Co^{60} source. The radiation was filtered by a half-inch lucite shield to remove the beta radiation. The intensity of radiation was 1000 r per minute. The controls were treated in the same manner, with the exception of the exposure to radiation. After radiation, control and treated suspensions were centrifuged, and the mitochondria re-suspended in one ml. This final suspension was assayed for phosphorylation.

For treatment with ultraviolet radiation the preparation was not diluted. Two and five-tenths ml. were placed in a quartz flask rotated in the ultraviolet beam by a slow speed stirring motor; 2.5 ml. were kept in a Pyrex tube in the cold as a control. During the period of irradiation, the quartz flask was partially submerged in an ice-water bath maintaining a temperature under 5° C. This flask was kept 11.5 cm. from a germicidal lamp for all radiations. The intensity of the radiation was 5500 ergs/cm²/sec. For most studies the radiations were filtered by a Corning No. 7910 filter which transmits principally those wave-lengths above 2400 Å (Filter I). In one case, to test the effect of longer wave-lengths, a Corning No. 9700 filter (transmits principally above 2700 Å) was used (Filter II). To test the effect of the flask rotation on phosphorylation, experiments were run in which the mitochondria were treated in the same way that they would have been for ultraviolet treatment with the exception that the ultraviolet source was not turned on.

For treatment with infrared radiation, the preparation was not diluted. Four or five ml. of the suspension were placed in a small test tube which was rotated in the infrared beam by a slow stirring motor. Temperature changes were eliminated by radiating the suspension while immersed in a water bath cooled by a Blue-M cooling coil. The bath was maintained at 9° C. for all studies. Controls were kept in the rear of the water bath wrapped in aluminum foil to prevent exposure to radiation; in two cases they were rotated as were the treated. The infrared source was 6 inches from the center of the exposed test tube. All radiation passed through a Corning No. 2540 filter, the glass side of the water bath (window glass), and 1

cm. of water. This arrangement provides intense radiation in the region 7600 to 15,000 Å. The source of radiation was a 250-watt commercial heat lamp operated at 110 volts. The usual exposure was for three hours. The intensity of the source is not accurately determined, but this was capable of increasing the chromosome aberrations in Tradescantia by at least 100% when used in combination with gamma rays.

For studies of the effects of combined radiations, the procedures outlined above were followed. The controls were taken through all the steps with the exception of exposure to radiation.

Some attempts to reactivate the ultraviolet-damaged phosphorylation mechanism were made. In attempts to photoreactivate the system, the suspension was exposed to visible light from a 250-watt incandescent spot-light filtered to remove the red wave-lengths. This treatment was carried out at 10° C. in the same water bath used in the infrared studies, to prevent heat-inactivation of the system. Treatments were of 20 minutes duration. In attempts to reactivate the system by the addition of co-factors, the co-factor was added to the reaction flask prior to assay of phosphorylation and oxygen uptake. In one case, 0.1 ml. of 0.01 *M* DPN was added, and in the other, 0.1 ml. of 10^{-4} *M* FAD.

Estimation of phosphorylation was conducted by a modification of the method of Maley and Lardy (1954), using succinate as the substrate. The main compartment of the Warburg vessel contained: 0.3 ml. (30 μM) phosphate buffer (pH 7.4), 0.3 ml. 0.1 *M* sodium succinate, 0.8 ml. 8.5% sucrose, 0.1 ml. (0.3 μM) cytochrome-*c*, 0.3 ml. (6 μM) ATP (Schwartz, neutral), 0.1 ml. (30 μM) $MgSO_4$, 0.1 ml. (40 μM) KF, and 0.5 ml. of the mitochondrial suspension. The center well contained 0.1 ml. 5 N KOH, and the side arm held 0.5 ml. (10 mg.) of hexokinase (Pabst) dissolved in 0.15 *M* glucose. To assure that the initial pH of the reaction would be 7.4 or higher, the pH of some of the more acid reactants was adjusted with NaOH before addition to the flasks. Readings of the oxygen uptake were taken for 30 minutes, after which the reactions were stopped with TCA and the phosphate determined by the Lowry-Lopez method as presented by Glick (1949). All Warburg assays were carried out at 25° C. All assays were made in duplicate. All experiments were repeated at least three times.

Results

The data presented in Table I show the effectiveness of ultraviolet radiation in the inactivation of oxidative phosphorylation. Although the phosphorylation mechanism is very labile and therefore shows variation from experiment to experiment and from assay to assay, it is clear that ultraviolet radiation inactivates phosphorylation rather easily. These data cannot be used to construct an inactivation curve because of the variability, but there is clear indication that the inactivation proceeds in a non-linear manner. It is also clear that the inactivation is not the result of either the time lag between the isolation of the mitochondria and the assay or the rotation in the quartz flask. Thirty minutes' rotation causes no loss of phosphorylation. Further, it can be seen that the filter which transmits principally those wavelengths above 2700 Å is not effective in bringing about this change. It would appear that the effective wave-length lies somewhere between the minimum of Filter I and the minimum of Filter II, that is, between about 2200 and 2700 Å.

TABLE I

Inactivation of phosphorylation by ultraviolet radiation

Treatment*	No. runs	Phosphate uptake (μM)		% Decrease	% Decrease O_2
		Controls	Treated		
5 min. UV	3	11.1 ± 2.1	10.2 ± 2.6	8.1	6.2
10 min. UV	3	12.7 ± 1.7	11.1 ± 2.0	12.6	3.5
20 min. UV	14	12.8 ± 1.1	7.4 ± 1.5	42.2	11.8
30 min. UV	3	15.7 ± 1.4	6.4 ± 0.8	59.2	3.0
30 min. rotation	3	13.8 ± 1.7	13.8 ± 1.7	0.0	0.6
30 min. UV Filter I	3	10.2 ± 2.6	1.8 ± 1.5	82.4	21.7
30 min. UV Filter II	3	10.2 ± 3.1	11.7 ± 2.7	−14.7	−11.6

* Particulates undiluted; treated in rotating quartz flask. Filter I transmits principally above 2400 Å; Filter II transmits principally above 2700 Å.

One other thing should be noted. The inactivation of the phosphorylating mechanism bears no clear relationship to the inactivation of the cytochrome oxidase system. Oxygen uptake is essentially unaffected in many cases in which there is extreme damage to phosphorylation. This is in agreement with earlier findings with gamma radiation (Yost and Robson, 1957).

The data in Table II show that the inactivated phosphorylation mechanism cannot be easily revived. Addition of DPN to the system stimulates phosphorylation but does not overcome the effect of the ultraviolet. As a matter of fact, when the

TABLE II

Failure to reactivate ultraviolet-damaged phosphorylation

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	4	10.3 ± 1.1	—	—
Control + DPN	4	13.7 ± 0.9	−33.0	− 7.7
20 min. UV	4	6.7 ± 1.5	35.0	16.4
20 min. UV + DPN	4	7.3 ± 0.8	29.1	5.7
20 min. UV + DPN using DPN as control			46.7	11.7
Control	3	21.2 ± 0.7	—	—
Control + FAD	3	20.2 ± 1.0	4.7	1.5
20 min. UV	3	16.2 ± 1.4	23.6	−10.4
20 min. UV + FAD	3	14.2 ± 1.0	33.0	−19.0
Control	5	15.7 ± 1.2	—	—
20 min. visible	5	15.1 ± 1.9	3.8	2.1
20 min. UV	5	11.1 ± 2.2	29.3	1.8
20 min. UV + 20 min. visible	5	10.1 ± 2.5	35.7	2.0

* Particulates undiluted; treated in a rotating quartz flask. Ultraviolet filtered by Filter I; visible light filtered to remove ultraviolet and infrared. DPH (0.1 ml. of 0.01 *M*) and FAD (0.1 ml. of 10^{-4} *M*) added after exposure.

DPN-treated series is used as a control for a series having both ultraviolet and DPN, the DPN-treated suspensions show greater inactivation than untreated. FAD has a more pronounced inhibitory effect. This may well result from the competition between the normal electron transport pathway in the mitochondrion and the FAD for excess electrons, when the system is partially damaged. Similarly, the system was not reactivated by light under our conditions. Treatment with visible light increased the damage in most cases even when filtered to remove far red and ultraviolet components. No attempt was made to use a narrow region of the visible near 3600 Å in these studies; however, the treatment used is similar to that used to reactivate bacteria successfully. Addition of cytochrome-*c* to the suspension did not result in reactivation by visible light.

Table III presents the data obtained in studies of the effect of infrared radiation on phosphorylation. It is important to note that this process is temperature-sensitive and that any rise in temperature during the infrared treatment results in inactivation

TABLE III

The effect of infrared radiation alone and in combination

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	3	13.8 ± 1.7	—	—
3 hrs. IR	3	14.0 ± 2.1	− 1.4	−2.2
20 min. UV	5	10.5 ± 1.5	23.9	4.2
3 hrs. IR + 20 min. UV	5	10.3 ± 2.0	25.4	5.1
Control	3	12.8 ± 1.5	—	—
20 min. UV	3	8.8 ± 1.0	31.2	6.7
20 min. UV + 3 hrs. IR	3	8.8 ± 1.0	31.2	5.9
Control	7	9.9 ± 2.5	—	—
2×10^4 r γ	10	5.4 ± 0.8	45.5	1.5
2×10^4 r γ + 3 hrs. IR	10	5.2 ± 0.8	47.5	−1.4

* Particulates undiluted for ultraviolet and infrared treatment; ultraviolet exposure in a rotating quartz flask; infrared exposure in a rotating 5-ml. test tube. Particulates diluted 1 in 20 for γ ray exposure.

of the phosphorylation mechanism. For this reason, the data reported here are those from studies in which the temperature was carefully controlled and kept below 10° C. Under these conditions, there is no effect of infrared treatment either alone or in combination with ultraviolet or gamma radiation. This statement must have one reservation, however; the variability between runs was rather high in these experiments as a result of the extended time of treatment and the dilution necessary for gamma radiation. As a result the averages may not present a completely accurate picture. In the case of pretreatment with infrared and treatment with ultraviolet radiation, three runs showed some increased inactivation and two showed decreased inactivation. There is a suggestion in these data that the infrared may have a slight effect on the phosphorylating mechanism, but nothing of the order of magnitude of its other known effects.

In Table IV are presented the data from studies of the effect of combined ultraviolet and gamma radiation. The figures in this table need explanation. The wide discrepancy between the two types of experiment with regard to the inactiva-

tion by ultraviolet alone is the result of the fact that to treat the mitochondria with gamma radiation the mitochondria must be diluted. Consequently in studies of the effects of post-treatment with ultraviolet the mitochondria had been diluted, centrifuged and re-suspended before treatment with ultraviolet. In this condition they seem more sensitive. Because of the difficulties of doing studies of this kind, these data can only be taken as *suggestive*. It would appear that the gamma radiation sensitizes the phosphorylation mechanism to ultraviolet treatment, but not the converse. However, unless these studies can be carried out in some manner which is not so drastic to the mitochondria, it will be impossible to be sure of this point. Attempts are being made to do this at present.

The oxidative phosphorylation of isolated particulates is sensitive to both aging and dilution of the preparation. However, in undiluted preparations kept in the cold (below 10° C.), the aging effects are slight for the first 4 or 5 hours. Therefore, most experiments reported in this paper were conducted under conditions in which the mitochondria were in fresh condition at the time of assay. Even the delay between isolation and assay of three hours, experienced with preparations

TABLE IV

The effect of combined ultraviolet and gamma radiation

Treatment*	No. runs	Phosphate uptake (μM)	% Decrease	% Decrease O_2
Control	3	11.8 ± 1.4	—	—
5 min. UV	3	9.2 ± 1.5	22.0	3.4
10^4 r γ rays	3	11.2 ± 1.0	5.0	− 5.6
5 min. UV + 10^4 r γ rays	3	9.2 ± 0.03	22.0	5.1
Control	3	8.7 ± 2.1	—	—
5 min. UV	3	3.3 ± 0.1	62.1	26.9
10^4 r γ rays	3	4.1 ± 0.9	52.9	22.4
10^4 r γ rays + 5 min. UV	3	2.0 ± 1.2	77.0	28.2

* Particulates undiluted for ultraviolet treatment; diluted 1 in 20 for γ ray treatment.

treated with infrared radiation, caused no appreciable decrease in phosphorylative ability due to aging. On the other hand, when a preparation is diluted, the phosphorylative ability is damaged. Therefore, the experiments done with gamma radiation show the superimposition of two effects: dilution and exposure to radiation. The degree of inhibition resulting from dilution varies from preparation to preparation. We are unable to explain this at the present time. This double effect in the gamma-treated series may be obscuring an effect that the radiation would have on undiluted preparations. The part of oxidative phosphorylation which is sensitive to dilution may be highly sensitive to radiation. However, this only means that the effects reported are the minimum effects of radiation. It should be emphasized that the effect of which we speak is a decrease in the total phosphate uptake and not a serious change in the P:O ratio. Some uncoupling occurs in highly diluted preparations but none occurs in undiluted preparations or in diluted preparations which are not allowed to stand in the diluted condition.

All the data presented in these tables represent the average values for the experiments, with standard errors. The problem of the variation from run to

run cannot be eliminated at this time. The mitochondria for each run are obtained from a different rat. Phosphate uptake and sensitivity to radiation varies from rat to rat. Attempts to standardize the procedures by using rats of uniform age and of defined diet reduce this variation only slightly. Therefore, all of these data must be regarded as indicating relationships in a manner that is qualitative rather than quantitative.

Discussion

The data presented in Table I show quite clearly that ultraviolet radiation in the wave-length region of 2400–2700 Å (probably 2600 Å) readily inactivates the oxidative phosphorylation mechanism of isolated cellular particulates. Furthermore, it is clear that the inactivation of phosphorylation is unrelated to inactivation of the oxidase system, since oxygen uptake may be very slightly affected in some cases in which phosphorylation is quite low. The separation of phosphorylation from oxidation is a common observation in mitochondrial preparations. Aging of the preparation will cause a loss of phosphorylative ability without appreciable damage to the oxidative system. Ultraviolet radiation merely accelerates this process. The observations with ultraviolet radiation are in complete agreement with those using gamma radiation (Yost and Robson, 1957). It appears that any one of a variety of agents will uncouple phosphorylation and oxidation.

The use of ultraviolet radiation to uncouple the phosphorylation mechanism presents some interesting approaches to the general problem of radiation effects and to the general problem of the sequence of events in oxidative phosphorylation. On this last point, Beyer (1958) has recently suggested that reversal of the effects of ultraviolet radiation may be used as a tool to indicate the normal pathway of electron flow in this system. His observation that addition of vitamin K is necessary to reactivate the glutamate system may be taken as evidence that this vitamin participates in the over-all process. Similar experiments may be done to elucidate further the mechanisms of phosphorylation. However, the observations of Beyer that photoreactivation can be achieved after treatment with ultraviolet radiation are not substantiated by the data presented in this paper. Under the conditions employed in these studies, it has been impossible to reactivate phosphorylation with visible light or the addition of several co-factors. Although the inactivation of oxidative phosphorylation by ultraviolet radiation may play a large part in the death of cells exposed to radiations, and although it is a well known fact that cells exposed to lethal doses of ultraviolet radiation can be revived by subsequent exposure to visible radiation (Kelner, 1949), the results reported in Table I indicate that the isolated particulate system cannot be reactivated by the same means that the whole cells can. Whether this inability to reactivate the phosphorylation mechanism is the result of a failure to use the right wave-lengths (3600 Å) exclusively, or is the result of failure to combine co-factor addition with light treatment can only be determined in the future. Whether the phosphorylation mechanism is inactivated in whole cells by ultraviolet radiation and then revived by light treatments will be the subject of a new series of investigations.

Since the report of inactivation and photoreactivation of DPN (Wells, 1956), it has seemed possible that any interference with oxidative metabolism might be the result of inactivation of the nucleotide-containing co-enzymes. These were tried

in this system in attempts to reactivate phosphorylation although they have no known function in the succinate system. That they failed to reactivate the system is not surprising. It does indicate that the decrease in phosphorylative ability is not the result of inactivation of some other (nucleotide-requiring) part of the system. Since ATP is a normal part of the reaction mixture, it is safe to assume that the effect is not upon the adenosine nucleotides. This agrees with the findings of Beyer (1958).

The data in Tables III and IV represent an attempt to find a mechanism of action for combined radiation effects. The effects of combined radiations on chromosomes must have their explanation either in the alteration of the ability of the breaks to rejoin or in the alteration of the fragility of the chromosome to radiation. Unless a mechanism of action can be discovered, it is unlikely that any satisfactory explanation will be forthcoming. The many experiments indicating that the damage to chromosomes by radiation is greatly influenced by known inhibitors of phosphorylation (Wolff and Luippold, 1955) suggests that studies on the combined effects of radiations on phosphorylation might give some important clues to radiation mechanisms. The data gathered in these experiments indicate that there is no pronounced effect of combined treatments with ultraviolet, infrared and gamma radiations. The potentiating effect of infrared, so clearly demonstrated in Tradescantia (Swanson, 1949), cannot be found in the mitochondrial systems; nor is there any indication of interactions (other than an additive effect of the radiations) between gamma rays and ultraviolet. These findings are not in accord with the report by Gordon and Surrey (1958) that near-infrared radiation accelerates the decay of phosphorylative ability in rat liver mitochondria. The techniques employed in the two studies are quite different and the difference in results is undoubtedly attributable to the greater sensitivity of the technique of Gordon and Surrey. Indeed this may explain the variability of some of our results. It is possible that a slight infrared effect is present in our test materials, but that our test is too insensitive to give a consistent measure of its magnitude. However, there can be little comparison of an effect on phosphorylation of this small degree with the enormous effect of infrared radiation on chromosome breakage. It seems highly unlikely that an increase in x-ray breakage of chromosomes amounting to 100 to 200 per cent can be induced by alterations of the phosphorylation mechanism too slight to be detected by the hexokinase method employed in this study.

Summary

Data are presented which show that ultraviolet radiation in the 2600 Å region inactivates oxidative phosphorylation. The addition of co-factors and the exposure to light of the preparations failed to reactivate the phosphorylation after treatment with ultraviolet. Treatment with infrared radiation and treatment with combined infrared and ultraviolet, infrared and gamma, and ultraviolet and gamma radiation failed to show any alteration in the normal inactivation patterns found with treatment with ultraviolet or gamma radiation alone. These data suggest that both ionizing and non-ionizing ultraviolet radiations damage cells, at least in part, by inactivation of oxidative phosphorylation. However, these data do not give any clue to the mechanism of action of combined radiations in altering the chromosomal structure of cells.

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